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User Manual

OriCell™ Dog Adipose-Derived Mesenchymal Stem Cells (ADSCs)

Cat. No. CAXMD-01001

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CONTENTS AND STORAGE

Product Name	Dog Adipose-derived Mesenchymal Stem Cells
Catalog No.	CAXMD-01001
Amount per Vial	1×10^6 Cells
Cryopreserved At	Second Passage
Storage Condition	Liquid Nitrogen

CAUTION: **Please handle this product as a potentially biohazardous material. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material, in the freezing medium.**

PRODUCT INTRODUCTION

Adipose-Derived Mesenchymal stem cells (ADSCs) are multipotent stem cells that can differentiate into a variety of cell types including osteocytes, adipocytes, and chondrocytes. ADSCs proliferate quickly and are capable of generating a local immunosuppressive microenvironment, thus contributing to their wide application potentials in tissue engineering, cell therapy, and gene therapy.

OriCell™ Dog Adipose-Derived Mesenchymal Stem Cells are derived from the adipose tissue at inguen of newborn healthy Beagle dogs. These cells have a strong capacity for self-renewal while maintaining their multipotency.

In addition, these cells have been tested for:

- Exogenous Factors: bacterial/fungal contamination, mycoplasma contamination, and endotoxin contamination.
- Characteristics: post-thaw viability, cell cycle, verification of undifferentiated state, and differentiation potential.

This product is intended for laboratory research use only. It is not intended for diagnostic, therapeutic, clinical, household, or any other applications.

CELL CHARACTERISTICS AND IDENTITY

- Strong capacity to expand. Can be passaged at least 5 times.
- Multipotent differentiation ability along the osteogenic, chondrogenic, and

adipogenic lineages.

PRODUCT APPLICATIONS

Dog ADSCs have become a popular research target due to their potential use in regenerative medicine and tissue engineering (in areas such as cardiovascular, neural, and orthopedic disease).

OriCell™ Dog ADSCs can be used as cell models to evaluate the immunoreactions, proliferation, immigration, and differentiation of ADSCs both *in vivo* and *in vitro*.

GENERAL HANDLING PRINCIPLES

1. Aseptic handling of the product is necessary throughout.
2. Once the cells have been established, always freeze up several vials of OriCell™ Dog ADSCs as a backup.

Note: **The OriCell™ DOG ADSCs can be frozen/thawed at least twice.**

3. For general maintenance of cells, we recommend the seeding density to be 2.0-2.5×10^4cells/cm^2.
4. For all studies, it is strongly recommended to use cells that are at, or under, an original passage number of 10.
5. For general maintenance of cells, we recommend that the medium is changed if it becomes acidic (the pH indicator in medium appears yellow). In general, change the growth medium every three days.
6. Do not let OriCell™ DOG ADSCs overgrow as it will result in contact inhibition. When the cells are 80-90% confluent, subculturing the cells is strongly recommended.

Note: **We strongly recommend the use of OriCell™ culture media and other related reagents for optimal results.**

THAWING AND ESTABLISHING OriCell™ DOG ADSCs

Materials Required

- OriCell™ Adipose-Derived Stem Cell Growth Medium (Cat. No. GUXMD-90011)

Thawing and Establishing Dog ADSCs

1. Pre-warm the fully supplemented (complete) OriCell™ ADSC Growth Medium to

37°C.

2. Add 9 mL of OriCell™ ADSC Growth Medium to a 15 mL conical tube.
3. Remove the cryovial of OriCell™ Dog ADSCs from liquid nitrogen.
4. Quickly thaw the cryovial in a 37°C water bath until the last ice crystal disappears. For optimal results, be sure to finish the thawing procedure within 3 minutes. Be careful not to submerge the entire vial. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells.

Note: **Results will be less than optimal if the cells are thawed for more than 3 minutes.**

5. As soon as the cells are completely thawed, disinfect the outside of the cryovial with 70% v/v ethanol.
6. Use a pipette to transfer the cells to the 15 mL conical tube containing OriCell™ ADSC Growth Medium inside a biosafety cabinet. Be careful not to introduce any bubbles during the transfer process.
7. Rinse the vial with 1 mL of the medium to reduce cell loss. Subsequently transfer this 1 mL of cell suspension into the conical tube.
8. Gently mix the cell suspension by slowly pipetting up and down. Be careful not to introduce any bubbles.
9. Centrifuge the cell suspension at 250 x *g* for 5 minutes.
10. Carefully aspirate off as much of the supernatant as possible and add 2-3 mL of fresh OriCell™ ADSC Growth Medium (pre-warmed to 37°C).
11. Gently resuspend the cells in OriCell™ ADSC Growth Medium.
12. Seed the cells into a T25 flask and add a sufficient amount of OriCell™ ADSC Growth Medium. Gently rock the culture flask to evenly distribute the cells.
13. Incubate the flask at 37°C inside a 5% CO_2 humidified incubator.
14. The next day, change the medium with fresh growth medium (pre-warmed to 37°C).
15. Change the growth medium every two days until the cells are 80% confluent thereafter.
16. When the cells are approximately 80-90% confluent, they can be dissociated with 0.25%Trypsin-0.04%EDTA and passaged.

Note: Changing Medium

1. **Warm an appropriate amount of medium to 37°C in a sterile container. Replace the spent medium with the pre-warmed, fresh medium. Once completed, return the flask to the incubator.**
2. **Avoid repeated warming and cooling of the medium. If the entire content is not needed for a single procedure, transfer only the required volume to a sterile secondary container.**

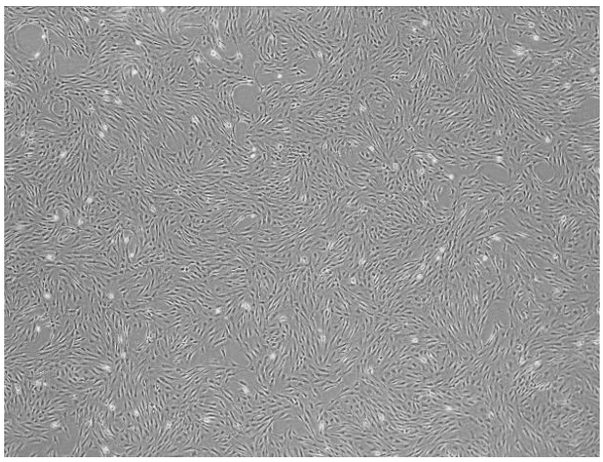


Fig. 1 OriCell™ Dog Adipose-Derived Mesenchymal Stem Cells are established.

PASSAGING OriCell™ DOG ADSCs

Materials Required

- 0.25%Trypsin-0.04%EDTA (Cat. No. TEDTA-10001)
- Phosphate-Buffered Saline (1×PBS) (Cat. No. PBS-10001)
- OriCell™ Dog Adipose-Derived Stem Cells (Cat. No. CAXMD-01001)
- OriCell™ Adipose-Derived Stem Cell Growth Medium (Cat. No. GUXMD-90011)

Passaging OriCell™ Dog ADSCs

1. Pre-warm the OriCell™ ADSC Growth Medium, 1×PBS, and 0.25%Trypsin-0.04%EDTA solution to 37°C.
2. Carefully aspirate the spent medium from the 80-90% confluent monolayer of ADSCs.
3. Add 1×PBS (6 mL for T75 flask, 3 mL for T25 flask). Be careful not to disturb the monolayer. Gently rock the flask back and forth to rinse the monolayer.
4. Aspirate 1×PBS off and discard.
5. Repeat steps 3-4 two or three times.
6. Add 0.25%Trypsin-0.04%EDTA solution (2-3 mL for T75 flask, 1 mL for T25 flask). Gently rock the flask back and forth to ensure that the entire monolayer is covered with the 0.25%Trypsin-0.04%EDTA solution. Allow trypsinization to continue until the majority of the cells (approximately 80%) are rounded up. At this point, gently tap the side of the flask to release the majority of cells from the culture flask surface.

Important: **Avoid leaving cells exposed to the trypsin longer than necessary (no more than two minutes if using Cyagen's trypsin-EDTA solution). Care should also be taken that the cells are not forced to detach prematurely as this may result in clumping.**

7. After the cells are visibly detached, immediately add the pre-warmed OriCell™ ADSC Growth Medium (6 mL for T75 flask, 3 mL for T25 flask) to neutralize the trypsinization.
8. Gently pipette the medium over the cells to dislodge and resuspend the cells. Repeat 5-6 times until all the cells are dissociated from the flask and evenly dispersed into a single cell suspension.
9. Transfer the dissociated cells into a 15 mL conical tube.
10. Centrifuge at 250 x *g* for 5 minutes.
11. Carefully aspirate off as much of the supernatant as possible.
12. Add 2 mL of OriCell™ ADSC Growth Medium to the conical tube and gently resuspend the cells thoroughly.
13. Plate the cells into appropriate flasks. OriCell™ Dog ADSCs can be split at 1:2 or other appropriate ratios.
14. Add an appropriate amount of medium to the cells. Incubate the cells at 37°C inside a 5% CO_2 humidified incubator.

Note: **Care should be taken to avoid introducing bubbles during pipetting.**

Additional Tips

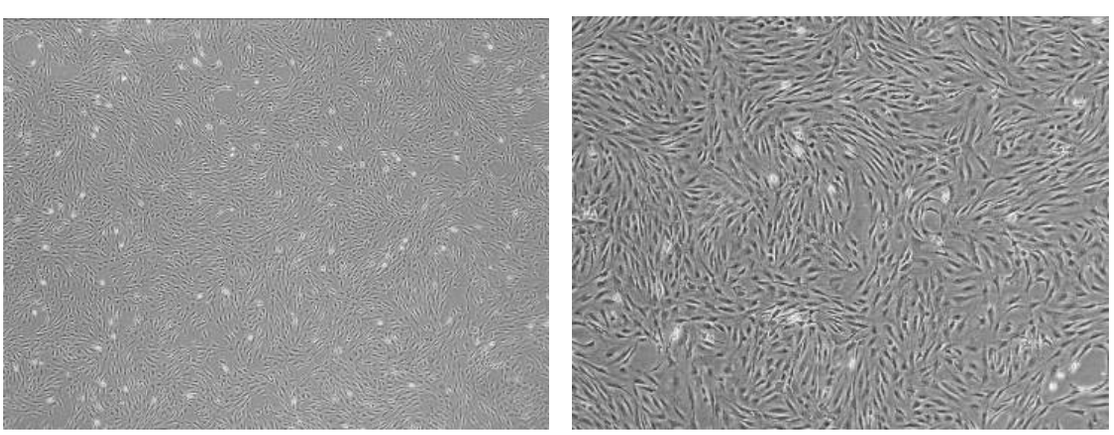
Time to Change Medium

It is recommended to change the culture medium if there are too many dead cells after passaging.

It is recommended to change the culture medium whenever the medium becomes acidic, even if the cells do not reach 80-90% confluency. The pH indicator in the culture medium will appear yellow when acidic. In general, change the growth medium every three days.

Time to Subculture

When OriCell™ Dog MSCs are 80-90% confluent, it is recommended that the cells be subcultured. Do not let the cells overgrow as it will result in contact inhibition.



Passage 5-40x Passage 5-40x

Fig. 2 Images of OriCell™ Dog Adipose-Derived Mesenchymal Stem Cells at passage 5

OriCell™ DOG ADSC DIFFERENTIATION USING OriCell™ DIFFERENTIATION MEDIA

OriCell™ Dog ADSCs can differentiate into a variety of cell types including osteocytes, adipocytes, and chondrocytes.

Osteogenic Differentiation

Materials Required

OriCell™ Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cat. No. GUXMX-90021)

Osteogenesis Protocol

***Note*: The protocol listed below is for 6-well tissue culture plates.**

1. Culture the OriCell™ Dog ADSCs in OriCell™ Mesenchymal Stem Cell Growth Medium at 37°C in a 5% CO_2 humidified incubator.
2. When cells are approximately 80-90% confluent, they can be dissociated with 0.25%Trypsin-0.04%EDTA (Cat. No. TEDTA-10001).
3. Reseed the MSCs in the growth medium at 2×10^4 cells/cm^2 in a 6-well tissue culture plate pre-coated with 0.1% gelatin solution.
4. Incubate the cells at 37°C in a 5% CO_2 humidified incubator.
5. When cells are approximately 60-70% confluent, carefully aspirate off the growth medium from each well and add 2 mL of OriCell™ Mesenchymal Stem Cell Osteogenic Differentiation Medium.
6. Feed cells every 3 days for 2-3 weeks by completely replacing the medium with fresh OriCell™ Mesenchymal Stem Cell Osteogenic Differentiation Medium (pre-warmed to 37°C).
7. After 2-3 weeks of differentiation, cells can be fixed and stained with alizarin red S.

***Note*: To prevent osteoblasts from detaching, it is recommended to change half of the medium every two days before analysis.**

Alizarin Red S Staining Analysis

1. After the cells have differentiated, remove the osteogenic differentiation medium from the wells and rinse with 1x phosphate-buffered saline (PBS). Fix cells with 2 mL of 4% formaldehyde solution for 30 minutes.
2. Rinse wells twice with 1x PBS. Stain the cells with 1 mL alizarin red S working solution for 3-5 minutes.
3. Rinse wells 2-3 times with 1x PBS.
4. Cells can now be visualized and analyzed under a microscope.

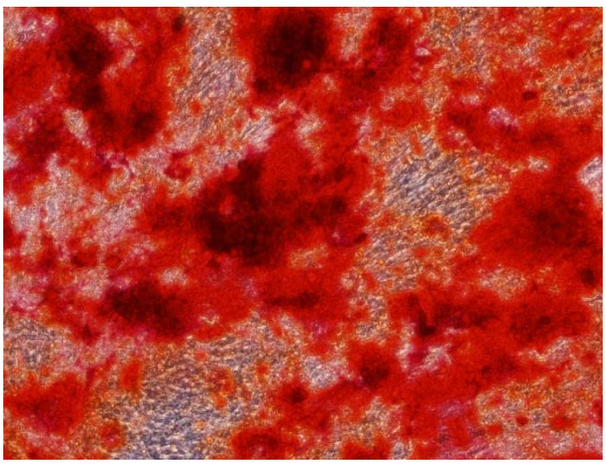


Fig. 3 OriCell™ Dog ADSCs are differentiated into Osteocytes and are stained with Alizarin Red S.

Adipogenic Differentiation

Materials Required

OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation Medium (Cat. No. GUXMX-90031)

Adipogenesis Protocol

***Note*: The protocol listed below is for 6-well tissue culture plates.**

1. Culture the OriCell™ Dog ADSCs in the OriCell™ Mesenchymal Stem Cell Growth Medium at 37°C in a 5% CO_2 humidified incubator.
2. When cells are approximately 80-90% confluent, they can be dissociated with 0.25%Trypsin-0.04%EDTA (Cat. No. TEDTA-1000).
3. Reseed the ADSCs in growth medium at $2x10^4$ cells/cm^2 in a 6-well tissue culture plate with a medium volume of 2 mL per well.
4. Incubate the cells at 37°C in a 5% CO_2 humidified incubator.
5. Feed the cells every three days until they are 100% confluent or post-confluent. Induction of adipogenic differentiation at post-confluency is strongly recommended.
6. When the cells are 100% confluent or post-confluent, carefully aspirate off the spent growth medium from the wells and add 2 mL of OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation medium A (induction medium) per well.
7. Three days later, change the medium to OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation medium B (maintenance medium) by completely replacing the spent medium A.
8. 24 hours later, change the medium back to MSC Adipogenic Differentiation medium A.
9. To optimally differentiate ADSCs into adipogenic cells, repeat the cycle of induction and maintenance three times.

10. After three to five cycles of induction and maintenance, culture the cells in OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation medium B for an additional 4-7 days until the lipid droplets are big, round enough. During these days period, change the medium every three days.

Oil Red O Stain Analysis

1. After the cells have differentiated, remove the MSC Adipogenic Differentiation Medium from the wells and rinse with 1x phosphate-buffered saline (PBS). Fix cells with 2 mL of 4% formaldehyde solution for 30 minutes.
2. Rinse wells twice with 1x PBS and stain cells with 1 mL of oil red O working solution (3:2 dilution with distilled water and filter with filter paper) for 30 minutes.
3. Rinse wells 2-3 times with 1x PBS.
4. Cells can now be visualized and analyzed under a microscope.

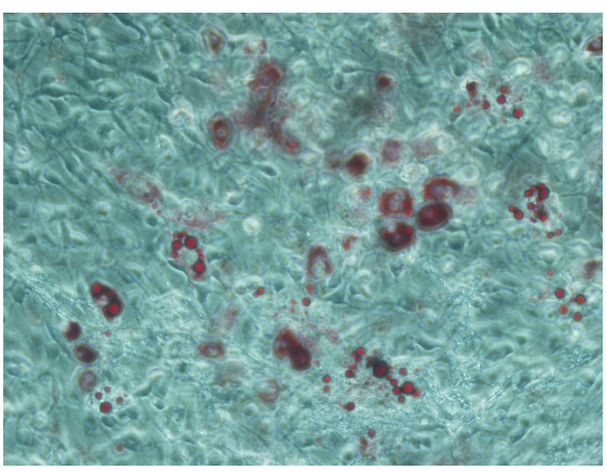


Fig.4 OriCell™ Dog ADSCs are differentiated into adipocytes and are stained with Oil Red O.

Chondrogenic Differentiation

Materials Required

OriCell™ Mesenchymal Stem Cell Chondrogenic Differentiation Medium

(Cat. No. GUXMX-90041)

Chondrogenesis Protocol

1. Calculate the total number of ADSC pellet cultures required for your experiment (2.5×10^5 ADSCs are needed to form each chondrogenic pellet). Transfer this amount of cells into an appropriate culture tube.
2. Wash the ADSCs with Incomplete Chondrogenic Medium. Centrifuge the cells at 150 x *g* for 5 minutes at room temperature, and then aspirate off the supernatant. Resuspend the cells in 1 mL of Incomplete Chondrogenic Medium per 7.5×10^5 cells. Centrifuge again at 150 x *g* for 5 minutes, and then aspirate off the medium.

3. Resuspend the ADSCs in Complete Chondrogenic medium to a concentration of 5.0×10^5 cells/mL.
4. Aliquot 0.5 mL (2.5×10^5 cells) of the cell suspension into 15 mL polypropylene culture tubes. Centrifuge the cells at 150 x *g* for 5 minutes at room temperature. DO NOT aspirate the supernatant or resuspend the pellet.
5. Loosen the caps of the tubes in order to allow gas exchange, and incubate the tubes at 37°C in a humidified atmosphere of 5% CO_2. Do not disturb the pellets for 24 hours.
6. Feed the cell pellets every 2-3 days by completely replacing the medium in each tube (to avoid aspirating the pellets when aspirating the medium, attach a sterile 1-200µL pipette tip to the end of the aspirating pipette). Add 0.5 mL of freshly prepared Complete Chondrogenic Medium to each tube.
7. After replacing the medium, flick the bottom of the tube to ensure that the pellet is free floating. Loosen the caps and return the tubes to the 37°C incubator.
8. Chondrogenic pellets should be harvested after 14-28 days in culture. Pellets may be formalin-fixed and paraffin-embedded for alcian blue stain analysis.

Alcian Blue Staining Procedure

1. The tissue sample should be formalin-fixed and paraffin-embedded already.
2. Staining procedure:
   a) Deparaffinize slides and hydrate to distilled water.
   b) Stain in alcian blue solution for 30 minutes.
   c) Wash in running tap water for 2 minutes.
   d) Rinse in distilled water.
   e) Visualize under a light microscope and capture images for analysis. Blue staining indicates synthesis of proteoglycans by chondrocytes.

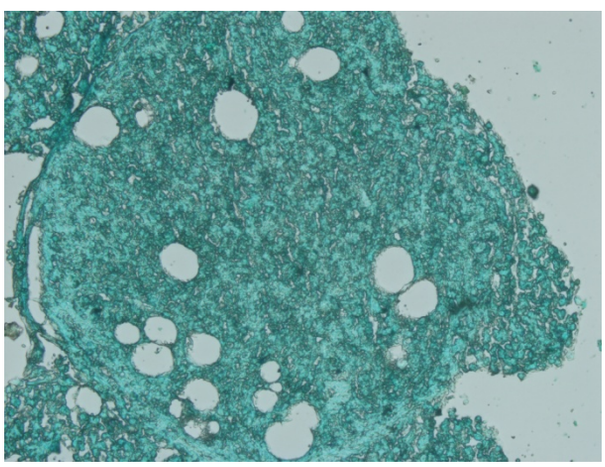


Fig.5 OriCell™ Dog ADSCs are differentiated into chondrocytes and are stained with Alcian Blue.

CRYOPRESERVATION OF CELLS USING OriCell™ CRYOPRESERVATION MEDIA

OriCell™ NCR Protein-Free Cryopreservation Medium (Cat. No. NCPF-10001) is a protein-free, ready-to-use freezing medium. Its chemically-defined and protein-free formulation has been optimized to stem cells and primary cells, thus greatly enhancing the viability and integrity of these cells by protecting them from damage during the one-step freeze-thaw procedure. Unlike other conventional freezing media, which require a slow programmed freeze, this product allows the cells to be directly frozen at -80°C.

Cryopreservation

Note: **Change the culture medium with fresh growth medium 24 hours before freezing.**

1. Collect cells that are in the logarithmic growth phase. Perform a cell count to determine the viable cell density.
2. Centrifuge the cells for 3-5 minutes at 250 x *g* and 20°C. Remove and discard the supernatant using a pipette.
3. Resuspend the cell pellet in the OriCell™ NCR Protein-Free Cryopreservation Medium at a cell density of 10^5-10^6 cells/mL.
4. Dispense aliquots of the cell suspension into cryogenic storage vials that are properly labeled.
5. Place the vials directly in a -80°C freezer. After 24 hours, transfer the frozen vials to liquid nitrogen for long-term preservation.

APPENDIX

Troubleshooting

The table below lists some potential problems and solutions for culturing ADSCs.

Problem	Cause	Solution
Low cell recovery rate	The storage condition does not meet the requirements	Purchase a replacement and store in liquid nitrogen for long-term preservation.
	Thawing of the cells takes too long	Thaw cells for no more than 3 minutes.
	Cells are incompletely recovered after thawing	After aspirating off medium, wash the tube with culture medium twice and transfer all of the cells to the dish.
	Cells are handled roughly	Care should be taken to avoid introducing bubbles during pipetting. Also avoid vortexing and high-speed centrifugation
	Medium is not pre-warmed	Warm medium to 37℃ before recovery.
Slow cell growth	Mycoplasma contamination	Discard the cells in question and disinfect the laboratory environment before recovering the next batch of cells.
	Over digestion	Wash the cells with PBS 2-3 times to remove serum prior to trypsinization (serum will inhibit the function of trypsin).
		Control the digestion time.
	Plating density is too low	Increase the plating density.
Cell aging	Inappropriate serum and medium	Use Cyagen tailor-made culture media. If other serum and media products are used, please perform validation to ensure compatibility.
	Dead cells are not removed promptly	Change the medium the next day after recovery to ensure removal of all dead cells.
	Cell Contamination	Discard the cells in question and disinfect the laboratory environment before recovering the next batch of cells.
	Plating density is too low	Some stem cells can secrete factors to support cell growth. Therefore, a certain degree of plating density must be maintained; otherwise, it will lead to cell proliferation slow down and cell aging.

Cell aging	Over digestion	Wash the cells with PBS 2-3 times to remove serum prior to trypsinization (serum will inhibit the function of trypsin).
		Control the digestion time.
	The passaging time is not appropriate	The cells should be subcultured when reaching 80-90% confluency in order to avoid contact inhibition.
Cells show spontaneous differentiation	DMSO is not completely removed during cell recovery	Wash the cells with pre-warmed medium 2-3 times during recovery.
Ineffective induction of cell differentiation	Differentiation reagents need to be optimized	Use Cyagen tailor-made differentiation media.
	Cell passage is too high	Use cells at a low original passage number.

Related Products

Product	Catalog Number
0.25%Trypsin-0.04%EDTA	TEDTA-10001
Phosphate-Buffered Saline (1xPBS)	PBS-10001
OriCell™ Dog Adipose-Derived Mesenchymal Stem Cells	CAXMD-01001
OriCell™ Dog Adipose-Derived Mesenchymal Stem Cell Growth Medium	GUXMD-90011
OriCell™ Mesenchymal Stem Cell Osteogenic Differentiation Medium	GUXMX-90021
OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation Medium	GUXMX-90031
OriCell™ Mesenchymal Stem Cell Chondrogenic Differentiation Medium	GUXMX-90041
OriCell™ NCR Protein-Free Cryopreservation Medium	NCPF-10001

REFERENCES

JM Gimble, and F Guilak. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *ISCT* 5: 362-369.

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